MAIL STOP APPEAL
BRIEF - PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	H. Lowenheim	Attorney Docket No. SOPH116953
Application No.:	09/622,719	Group Art Unit: 1635
Filed:	October 18, 2000	Examiner: K.A. Lacourciere
Title:	METHOD FOR THE TREATMENT OF DISEASES OR DISORDERS OF THE INNER EAR	

TRANSMITTAL OF APPEAL BRIEF

Seattle, Washington 98101

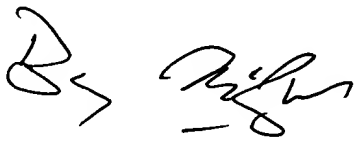
September 20, 2005

TO THE COMMISSIONER FOR PATENTS:

Enclosed herewith for filing in the above-identified application is an Appeal Brief. Also enclosed is Check No. 16080 in the amount of $250.00. The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17, and 1.18 which may be required during the entire pendency of the application, or credit any overpayment, to Deposit Account No. 03-1740. This authorization also hereby includes a request for any extensions of time of the appropriate length required upon the filing of any reply during the entire prosecution of this application. A copy of this sheet is enclosed.

Respectfully submitted,

CHRISTENSEN O'CONNOR
JOHNSON KINDNESS[PLLC]



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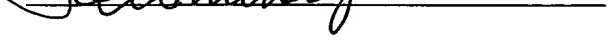
Date: September 20, 2005 

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BRIEF - PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	H. Lowenheim	Attorney Docket No. SOPH116953
Application No:	09/622,719	Group Art Unit: 1635 / Confirmation No.: 1261
Filed:	October 18, 2000	Examiner: T.A. Vivlemore
Title:	METHOD FOR THE TREATMENT OF DISEASES OR DISORDERS OF THE INNER EAR	

APPELLANT'S APPEAL BRIEF

Seattle, Washington
September 20, 2005

TO THE COMMISSIONER FOR PATENTS:

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TABLE OF CONTENTS

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I. REAL PARTY IN INTEREST

Sound Pharmaceuticals Incorporated, a Washington corporation, having a place of business at 4010 Stone Way N., Suite 120, Seattle, Washington 98103, is the assignee of the entire interest of the appealed subject matter.

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II. RELATED APPEALS AND INTERFERENCES

There are none.

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III. STATUS OF CLAIMS

Claims 28, 31, and 63 are pending in the application. All stand rejected under 35 U.S.C. § 112, first paragraph. Claims 28, 31, and 63 are appealed. The table below indicates their status.

Claim(s)	Status	Appealed
1-27	Canceled	No
28	Rejected	Yes
29-30	Canceled	No
31	Rejected	Yes
32-62	Canceled	No
63	Rejected	Yes
64-66	Canceled	No

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IV. STATUS OF AMENDMENTS

The application was rejected in an Office Action dated September 10, 2002. Thereafter, an Amendment and Response to the non-final Office Action was mailed on March 7, 2003, and entered into the file. An additional non-final Office Action was mailed on June 4, 2003. A further Amendment and Response to this Office Action was mailed on November 4, 2003, and entered into the file. The application was finally rejected in a paper dated February 13, 2004. An Amendment and Response After Final was mailed on May 11, 2004, but was not entered into the file. An Advisory Action was mailed on May 25, 2004. Thereafter, an Amendment and Response, together with a Request for Continued Examination, was mailed on June 9, 2004, and entered into the file. The application was again rejected in an Office Action dated July 2, 2004. Thereafter, a Response to Office Action was mailed on December 29, 2004, and entered into the file. The application was again finally rejected in a paper dated March 15, 2005. A copy of the pending claims is attached in the Claims Appendix.

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V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention relates to a process for the treatment of disease or disorders of the inner ear, which are caused by damage or destruction of the sensory cells of the inner ear. (See instant Specification, page 1, first paragraph.) Prior to the present invention, it was not possible to regenerate irreversibly damaged cells in the highly differentiated sensory epithelia in the inner ear of humans and other mammals. Thus, a partial or complete hearing loss due to damage or destruction of the sensory cells of the inner ear was generally irreversible. (See instant Specification, page 1, third paragraph.)

Inner ear sensory cells are located upon a layer of supporting cells. The supporting cells do not normally divide or regenerate in adult mammals. (See, e.g., instant Specification, page 2, second paragraph.) In the practice of the present invention, one or more cell cycle inhibitors in the supporting cells of the inner ear are inhibited, or eliminated, so that the supporting cells re-enter the cell cycle and divide, thereby creating cells which can differentiate to form new sensory cells and supporting cells. (See instant Specification, page 3, second paragraph.) The cell cycle inhibitor that is targeted in the practice of the currently claimed invention is a member of the so-called cyclin-dependent kinase inhibitors, called $p27^{kip1}$. (See instant Specification, page 4, second paragraph.)

In the practice of the currently claimed invention, antisense molecules are used to inhibit $p27^{kip1}$ synthesis. The antisense molecules are short nucleic acid molecules (sometimes referred to as oligonucleotides) that bind to mRNA that encodes $p27^{kip1}$, thereby blocking the synthesis of $p27^{kip1}$ in cells. (See instant Specification, page 5, second paragraph.)

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VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

First Ground of Rejection - Claims 28, 31, and 63

Claims 28, 31, and 63 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. This is a written description rejection.

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VII. ARGUMENT

Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 28, 31, and 63

The Examiner argues that Claims 28, 31 and 63 are drawn to methods that require antisense molecules targeted to any mammalian $p27^{kip1}$. The Examiner states that the specification does not disclose the structure (i.e. nucleotide sequence) of any antisense molecules targeted to mammalian $p27^{kip1}$, nor does it disclose the target sequences for any mammalian $p27^{kip1}$ or the common structural elements (e.g. regions of homology) for mammalian $p27^{kip1}$. The Examiner notes that the prior art at the time of the invention provided two antisense molecules targeted to one species of mammalian $p27^{kip1}$ (human $p27^{kip1}$) and disclosed the nucleotide sequence encoding three species of mammalian $p27^{kip1}$.

The Examiner further argues that the genus of mammalian $p27^{kip1}$ is broad, encompassing any mammalian organism and the species encompassed within the genus are highly variant (for example, with regard to nucleotide sequence.) The Examiner notes that, at the time of the invention, $p27^{kip1}$ from three mammals was known in the prior art, however, according to the Examiner, knowledge of three homologs of $p27^{kip1}$ is not sufficient to describe all homologs of $p27^{kip1}$ from all mammals. According to the Examiner, the specification does not correct the deficiencies of the prior art, because the prior art does not describe any homologs of $p27^{kip1}$ from any other mammal.

As a preliminary matter, applicant respectfully disagrees with the Examiner's assertion that the species encompassed by the genus of mammalian $p27^{Kip1}$ molecules is highly variant, for example with regard to nucleotide sequence. Applicant submits that, on the contrary, the art teaches that the species encompassed within the genus of mammalian nucleic acid molecules encoding $p27^{Kip1}$ proteins have highly related sequences. For example, in the response

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submitted on June 9, 2004, together with the Request for Continued Examination, applicant provided a Clustal W Alignment of the nucleic acid sequences encoding human, mouse, and mink $p27^{Kip1}$ proteins. The nucleic acid sequences encoding human, mouse, and mink $p27^{Kip1}$ proteins have been publicly available under GenBank accession numbers U10906, U09968 and U09966, respectively, since July 27, 1994. This alignment showed that these three nucleic acid sequences are more than 85% identical. A copy of the Clustal W Alignment is submitted herewith as Attachment A in the Evidence Appendix. Further, as described by Polyak et al. (*Cell 78*:59-66 (1994)), which was made of record in the response dated November 4, 2003, the $p27^{Kip1}$ proteins encoded by these nucleic acid molecules are about 90% identical (Polyak et al., page 61, last line, to page 62, line 2). A copy of the Polyak et al. publication is submitted herewith as Attachment B in the Evidence Appendix.

In the Office Action mailed March 15, 2005, in the section entitled "Response to Arguments", the Examiner states that,

> Applicant argues that the Examiner has not provided a basis for stating the genus of $p27^{Kip1}$ is variant with regard to nucleotide sequences and refers to previous arguments demonstrating 85 % homology between the three known mammalian $p27^{Kip1}$. This is not persuasive as this demonstrates that the known mammalian $p27^{Kip1}$ have variability of 15 %.

Applicant submits that sequences that are more than 85 % identical are highly related and homologous. Indeed, Applicant notes that the Court of Appeals for the Federal Circuit recognizes that nucleic acid sequences that are more than 85 % identical are highly homologous. For example, in *Enzo Biochem, Inc. v. GenProbe, Inc.*, (296 F.3d 1316, 63 U.S.P.Q. 2d 1609 (Fed. Cir. 2002)) the Court of Appeals for the Federal Circuit described the background to the case as follows:

> Enzo is the assignee of the '659 patent, which is directed to nucleic acid probes that selectively hyridize to the genetic material of the bacteria that cause gonorrhea, *Neisseria gonorrhoeae*. *N. gonorrhoeae* reportedly has

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> between eighty and ninety three percent homology with *Neisseria meningitidis*. '659 patent, col. 2, 11. 61-64. Such a high degree of homology has made detection of *N. gonorrhoeae* difficult, as any probe capable of detecting *N. gonorrhoeae* may also show a positive result when only *N. meningitidis* is present. *Enzo Biochem, Inc.*, 296 F.3d at 1320-1321, 63 U.S.P.Q. 2d at 1610. [Underline added.]

Thus, Applicant submits that the art teaches that the species encompassed by the genus of nucleic acid molecules encoding mammalian $p27^{Kip1}$ proteins are highly conserved and homologous.

With respect to the Examiner's argument that the instant specification, and the prior art, do not adequately describe the genus of nucleic acid molecules that encode a $p27^{kip1}$, Applicant notes that the written description requirement may be satisfied if in the knowledge in the art the disclosed function is sufficiently correlated to a particular, known, structure. *Amgen Inc., v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1332, 65 U.S.P.Q. 2d 1385, 1398 (Fed. Cir. 2003), citing *Enzo Biochem* 296 F.3d at 1324, 63 U.S.P.Q. 2d at 1398.

Applicant submits that the prior art discloses the nucleic acid sequences of mRNAs encoding human, mouse, and mink $p27^{Kip1}$ proteins (publicly available under GenBank accession numbers U10906, U09968 and U09966, respectively). As shown in the attached Clustal W alignment (Attachment A), the nucleic acid sequences of these three $p27^{Kip1}$ mRNAs are highly conserved. Applicant submits that it can be reasonably inferred that other mRNAs that encode a mammalian $p27^{Kip1}$ protein are highly conserved. Thus, Applicant submits that the function of encoding a $p27^{Kip1}$ protein is correlated with highly conserved nucleic acid sequences. Applicant submits, therefore, that the prior art has adequately described the genus of nucleic acid molecules that encode a mammalian $p27^{Kip1}$.

With respect to the $p27^{Kip1}$ antisense nucleic acid molecules, Applicant submits that the written description requirement is satisfied because knowledge of the sequence of a single member of the highly conserved family of $p27^{Kip1}$ genes is sufficient to allow one of ordinary

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skill in the art to make effective $p27^{Kip1}$ antisense nucleic acid molecules. As described more fully below, inventor Jonathan Kil made fourteen effective $p27^{Kip1}$ antisense nucleic acid molecules. The antisense nucleic acid molecules each corresponded to a different sequence within the first 445 bases of the target $p27^{Kip1}$ mRNA. Thus, one of ordinary skill in the art can readily determine the sequence of an effective $p27^{Kip1}$ antisense molecule by selecting a portion of a nucleic acid molecule that encodes $p27^{Kip1}$.

The antisense experiments conducted by Jonathan Kil are described in the declaration (referred to as the Third Kil Declaration) filed with the response dated December 29, 2004. A copy of the Third Kil Declaration is submitted herewith as Attachment C in the Evidence Appendix. A copy of Dr. Kil's *Curriculum vitae* is submitted herewith as Attachment D in the Evidence Appendix, and was made of record in the response dated June 9, 2004. The Third Kil Declaration describes the results of experiments in which 14 antisense oligonucleotides (directed against mouse $p27^{Kip1}$ mRNA) were introduced into mouse NIH 3T3 cells, cultured *in vitro*, and subsequently the level of $p27^{Kip1}$ mRNA was measured.

The nucleic acid sequences of the 14 antisense oligonucleotides are set forth in Table 1, paragraph 3, of the Third Kil Declaration. As described in paragraph 3 of the Third Kil Declaration, the location of each oligonucleotide is given with reference to the sequence of the mouse $p27^{Kip1}$ cDNA (GenBank accession number U09968; reported in Polyak, K., et al, *Cell 78*: 56-66 (1994)).

As described in paragraph 3 of the Third Kil Declaration, the cells were incubated in the presence of the oligonucleotide for 26 hours. Real time PCR was used to measure the amount of $p27^{Kip1}$ mRNA present in total RNA extracted from the treated cells.

Enclosed herewith as Attachment E is a graph showing the level of $p27^{Kip1}$ mRNA in the cells treated with the different oligonucleotides, compared to the control level of $p27^{Kip1}$ mRNA

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in cells treated with the Lipofectamine lipid delivery vehicle without oligonucleotides. A copy of this graph was filed with the response dated December 29, 2004. As described in paragraph 4 of the Third Kil Declaration, the results shown in the graph (which is referred to as Attachment B in the Third Kil Declaration) demonstrate that all of the tested oligonucleotides caused a significant reduction in the level of $p27^{Kip1}$ mRNA in the treated cells.

As can be seen from Table 1 of the Third Kil Declaration, the antisense oligonucleotides each corresponded to a different sequence within the first 445 bases of the $p27^{Kip1}$ mRNA. Thus, the results of these experiments are consistent with the view that the level of expression of a $p27^{Kip1}$ mRNA can be significantly reduced by an antisense oligonucleotide that corresponds to any sequence of at least 14 consecutive nucleotides within a $p27^{Kip1}$ mRNA. Applicant submits, therefore, that the written description requirement is satisfied by the existence, in the prior art, of the nucleic acid sequence of at least one member of the highly conserved genus of mammalian $p27^{Kip1}$ mRNAs (e.g., the sequence of the mouse $p27^{Kip1}$ cDNA, set forth in the GenBank database as accession number U09968, which was reported by Polyak et al. in 1994) that can be used as a source of antisense oligonucleotide sequences.

Consequently, Applicant requests withdrawal of the rejection of Claims 28, 31, and 63 under 35 U.S.C. § 112, first paragraph.

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VIII. CLAIMS APPENDIX

1-27. (Canceled)

28. (Currently Amended) A process for the treatment of hearing loss caused by damaged inner ear sensory hair cells, the process comprising the step of at least partly inhibiting or eliminating the action of $p27^{Kip1}$ present in the inner ear by local administration of antisense molecules to mammalian $p27^{Kip1}$ to the inner ear, thereby promoting regeneration of the sensory hair cells of the inner ear.

29-30. (Canceled)

31. (Previously presented) The process according to claim 28, characterized in that the regeneration of the sensory cells of the inner ear takes place by stimulating proliferation of the supporting cells of the inner ear.

32-62. (Canceled)

63. (Currently amended) A process for promoting regeneration and growth of sensory hair cells in the inner ear of a mammalian subject in need thereof, the process comprising the step of locally administering antisense molecules to mammalian $p27^{Kip1}$ to the inner ear in an amount sufficient to promote regeneration and growth of sensory hair cells in the inner ear.

64-66. (Canceled)

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IX. EVIDENCE APPENDIX

Appendix A	Clustal W Alignment
Appendix B	Polyak et al., *Cell 78*:59-66, 1994
Appendix C	Third Kil Declaration
Appendix D	*Curriculum vitae* of Dr. Jonathan Kil
Appendix E	Graph showing the level of p27Kip1 mRNA in cells treated with different oligonucleotides

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X. RELATED PROCEEDINGS APPENDIX

There are none.

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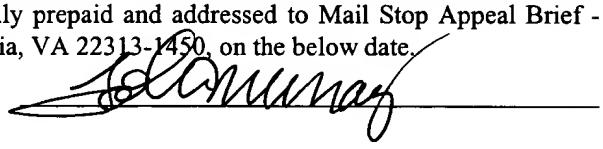
Date: September 20, 2005 

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CLUSTAL W (1.82) multiple sequence alignment

```
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gi|516545|gb|U09968.1|MMU09968     ATGTCAAACGTGAGAGTGTCTAACGGGAGCCCGAGCCTGGAGCGGATGGA 50
                                   *********** * ***************** ****************

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gi|516545|gb|U09968.1|MMU09968     CGCCAGACAAGCGGATCACCCCAAGCCTTCCGCCTGCAGAAATCTCTTCG 100
                                   ****** ** *****  ********** ** ******** ** *******

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gi|516545|gb|U09968.1|MMU09968     GCCCGGTCAATCATGAAGAACTAACCCGGGACTTGGAGAAGCACTGCCGG 150
                                   *******  * ** *****  * ********************** ** *

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gi|516543|gb|U09966.1|MVU09966     GACATGGAAGAGGCAAGCCAGCGCAAGTGGAATTTTGATTTCCAGAATCA 200
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gi|516545|gb|U09968.1|MMU09968     TAAGCCCCTGGAGGGCAGATACGAATGGCAGGAGGTGGAGAGGGGCAGCT 250
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gi|516545|gb|U09968.1|MMU09968     TGCCCGAGTTCTACTACAGGCCCCCGCGCCCCCCCAAGAGCGCCTGCAAG 300
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                                   **** ****************** ********** *** * *** * ***

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gi|516543|gb|U09966.1|MVU09966     CAACAGAACAGAAGAAAATGTCTCAGACGGTTCC---------------- 534
gi|516545|gb|U09968.1|MMU09968     CAACAGAACAGAAGAAAATGTTTCAGACGGTTCCCCGAACGCTGGCACTG 550
                                   ********************* ************

gi|48146914|emb|CR457399.1|        TGGAGCAGACGCCCAAGAAGCCTGGCCTCAGAAGACGTCAAACTTAA 597
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gi|516545|gb|U09968.1|MMU09968     TGGAGCAGACGCCCAAGAAGCCCGGCCTTCGA---CGCCAGACGTAA 594
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CLUSTAL W (1.82) Multiple Sequence Alignments

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Sequence 3: gi|516545|gb|U09968.1|MMU09968      594 bp
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Sequences (1:3) Aligned. Score:  87
Sequences (2:3) Aligned. Score:  88
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Group 2: Sequences:   3      Score:9773
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Cloning of $p27^{Kip1}$, a Cyclin-Dependent Kinase Inhibitor and a Potential Mediator of Extracellular Antimitogenic Signals

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Summary

We cloned $p27^{Kip1}$, a cyclin-dependent kinase inhibitor implicated in G1 phase arrest by TGFβ and cell-cell contact. $p27^{Kip1}$ associates with cyclin E-Cdk2 complexes in vivo and in vitro, prevents their activation, and inhibits previously activated complexes, and $p27^{Kip1}$ overexpression obstructs cell entry into S phase. $p27^{Kip1}$ potently inhibits Rb phosphorylation by cyclin E-Cdk2, cyclin A-Cdk2, and cyclin D2-Cdk4. $p27^{Kip1}$ is highly conserved and broadly expressed in human tissues, and its mRNA levels are similar in proliferating and quiescent cells. $p27^{Kip1}$ has a region of sequence similarity to $p21^{Cip1/WAF1}$, the Cdk inhibitor whose transcription is stimulated by p53. A $p27^{Kip1}$ peptide corresponding to this region retains Cdk inhibitory activity. We suggest that cell contact, TGFβ, and p53 all restrain cell proliferation through related Cdk inhibitors.

Introduction

Cell cycle transitions are orchestrated by cyclin-dependent kinases that consist of a catalytic subunit called Cdk and an activating subunit called cyclin, paired in diverse combinations (Hartwell, 1992; Nurse, 1990). In mammalian cells, the cyclin E-Cdk2 and cyclin D-Cdk4 complexes are catalytically active during G1 phase and rate limiting for cell progression through this period, and one of their putative substrates is Rb whose phosphorylation is required for cell transition into S phase (Sherr, 1993, and references therein). Active cyclin A-Cdk2 complexes appear during the G1/S transition (Sherr, 1993), and complexes of Cdc2 (Cdk1) with cyclins A and B become active in G2 and are required for mitosis (Nurse, 1990).

G1 cyclin-dependent kinases act as integrators of positive and negative signals that determine progression towards a point in late G1 beyond which the cell cycle proceeds autonomously (Pardee, 1989). Some of these signals regulate the protein levels of cyclin-dependent kinases (Cocks et al., 1992; Ewen et al., 1993; Geng and Weinberg, 1993; Matsushime et al., 1991). Another level of regulation results from the action of Cdk-inhibitory proteins. One of these is Cip1/WAF1 (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993), which is transcriptionally activated by p53 (El-Deiry et al., 1993) and by cell senescence (Noda et al., 1994). p16 is a Cdk4 inhibitor originally identified by virtue of its association with Cdk4 in certain transformed cell lines (Serrano et al., 1993). Candidate Cdk inhibitors have been identified in yeast as well. In Saccharomyces cerevisiae, mating factors arrest cell cycle through *FAR1* (Chang and Herskowitz, 1990), a gene whose product binds to CLN-CDC28 complexes (Peter et al., 1993). Another CDC28 inhibitor is $p40^{SIC1}$ (Nugroho and Mendenhall, 1994). The *rum1* gene that controls G1 progression in Schizosaccharomyces pombe also encodes a candidate Cdk inhibitor (Moreno and Nurse, 1994). As an emerging group of negative cell cycle regulators, the Cdk inhibitors are of interest because their disruption would deprive cells of critical antiproliferative devices, thereby contributing to cancer. This possibility has been recently substantiated with the finding that the p16 gene may correspond to the multiple tumor suppressor 1 (*MTS-1*) locus of human chromosome 9p21 and is deleted or mutated in various human malignancies (Kamb et al., 1994; Nobori et al., 1994).

Studies on the mechanism of mammalian cell cycle arrest by extracellular signals have indicated that Cdk inhibitors are involved in this process, too. The observation that TGFβ and cell-cell contact prevent activation of the cyclin E-Cdk2 complex during G1 phase (Koff et al., 1993) led to the identification of Kip1, a heat-stable protein of 27 kDa present in extracts from cells made quiescent by these signals (Polyak et al., 1994). Kip1 binds tightly to cyclin E-Cdk2 and cyclin D-Cdk4 complexes and inhibits their Cdk activity in vitro in a stoichiometric manner (Polyak et al., 1994). Here, we describe the isolation, molecular cloning, and characterization of Kip1. We show that Kip1 is structurally related to Cip1/WAF1, thus defining a family of Cdk inhibitors whose members may mediate diverse antiproliferative signals.

Results

Purification and Cloning of Kip1

Lysates from contact-inhibited Mv1Lu cells were heated to 100°C, cleared of insoluble material, and allowed to bind to a cyclin E-Cdk2 affinity column. Elution with 6 M guanidium hydrochloride yielded recombinant Cdk2 released from the column and the 27 kDa protein Kip1. Dialyzed aliquots of this sample had strong inhibitory activity towards cyclin E-Cdk2 in histone H1 kinase assays, and this activity was previously shown to coelute from SDS-polyacrylamide gel slices with Kip1 (Polyak et al., 1994). The Kip1 yield from two separate preparations ($\sim 2 \times 10^{10}$ cells each) was 0.3 μg and 1 μg, respectively.

To confirm that Kip1 interacts with Cdk2 in vivo, we immunoprecipitated metabolically labeled extracts from contact-inhibited Mv1Lu cells using anti-Cdk2 antibodies (Figure 1B). In addition to Cdk2, the precipitate contained a 27 kDa band whose peptide map, after limited digestion

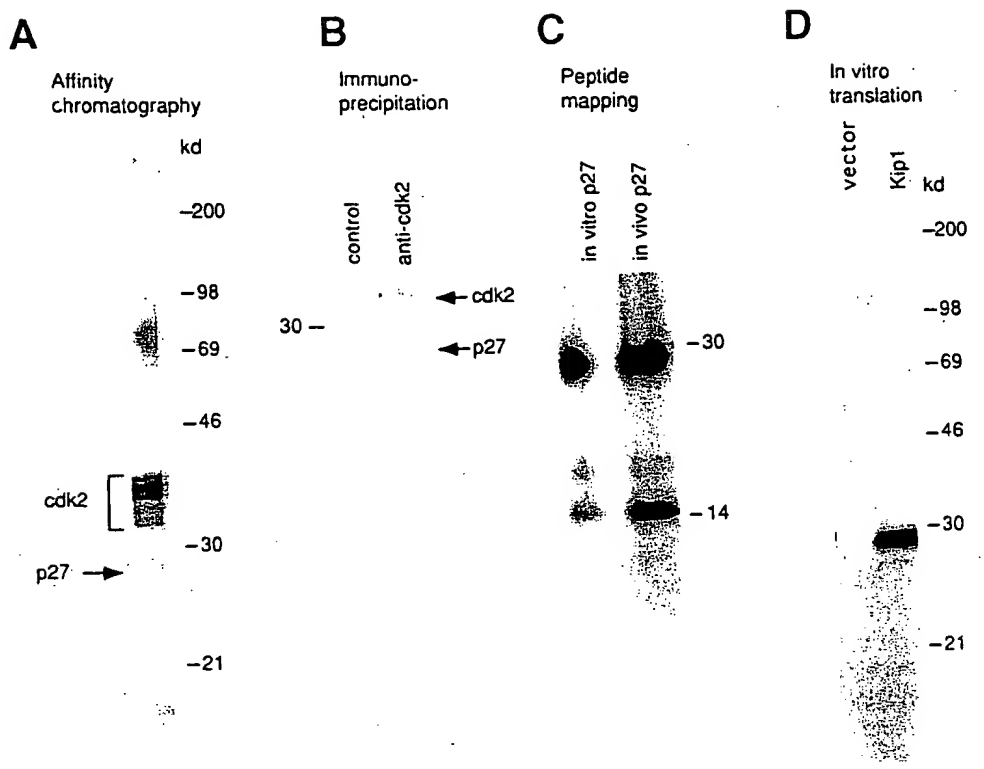


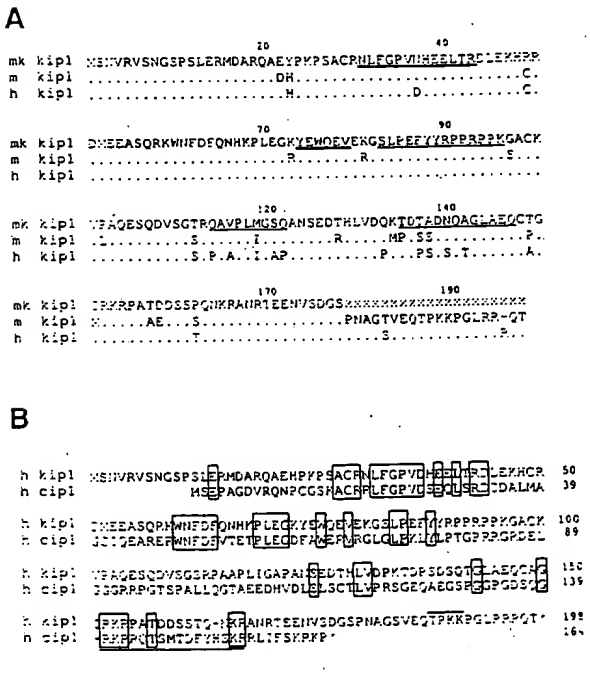
Figure 1. Purification, Cyclin E–Cdk2 Interaction, and In Vitro Translation of Kip1

(A) Heat-treated extracts from quiescent Mv1Lu cells were subjected to cyclin E–Cdk2 affinity chromatography. The eluate was resolved by SDS-PAGE and silver stained. $p27^{Kip1}$ is indicated by an arrow. The broad band is Cdk2–hemagglutinin, and the 69 kDa band is a contaminant present also in blank lanes.

(B) Extracts from metabolically labeled quiescent Mv1Lu cells were precipitated with preimmune rabbit serum (control) or anti-Cdk2 antibody.

(C) Metabolically labeled p27 obtained by coprecipitation with anti-Cdk2 antibodies as in (B) (in vivo p27) or by cyclin E–Cdk2 affinity chromatography as in (A) (in vitro p27) was digested with V8 protease and displayed by SDS-PAGE and fluorography.

(D) In vitro translations containing empty vector (vector) or vector encoding histidine-tagged mouse Kip1 (Kip1) were bound to Ni^{2+}–NTA–agarose, boiled in sample buffer, and resolved by SDS-PAGE.



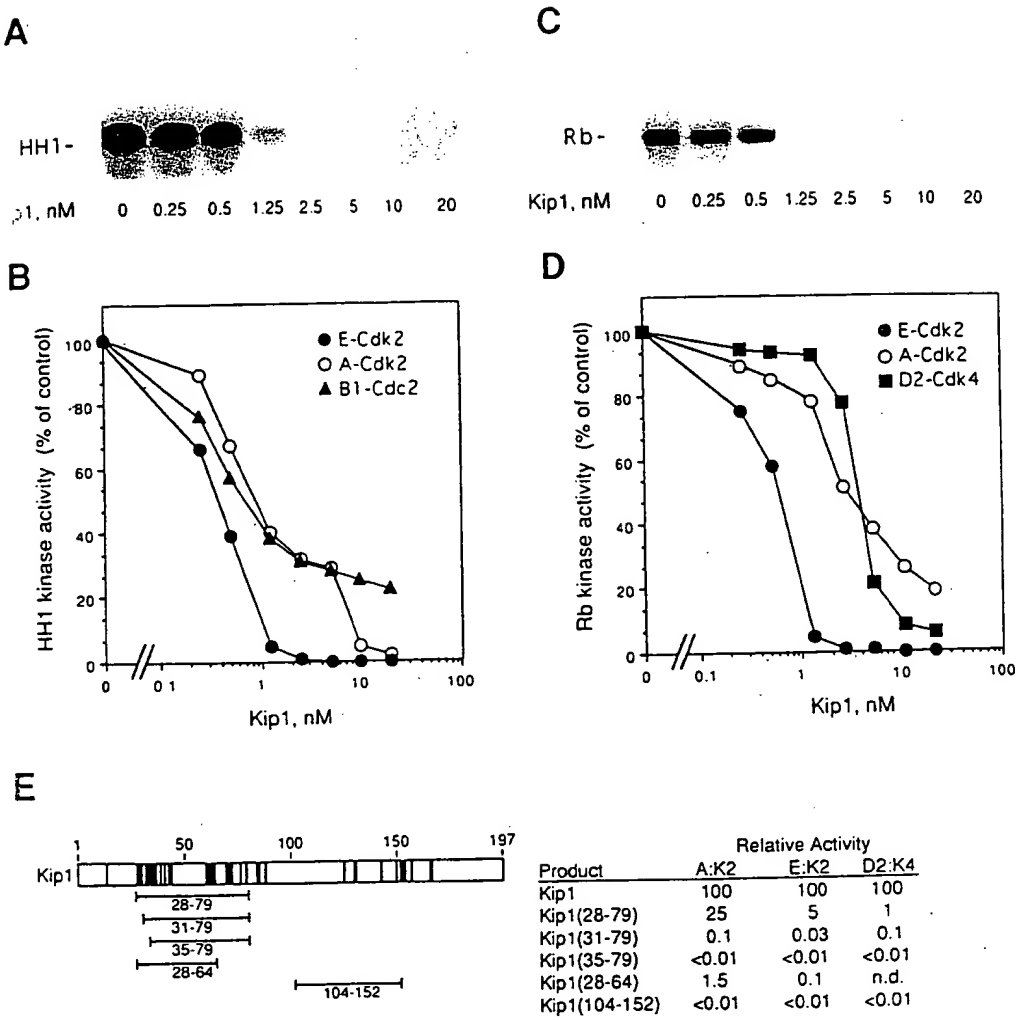
with V8 protease, was identical to that of Kip1 purified from metabolically labeled cells by cyclin E–Cdk2 affinity chromatography (Figure 1C). These results provided further evidence that the Cdk inhibitor purified by binding to cyclin E–Cdk2 in vitro was associated with Cdk2 in quiescent cells.

Various Kip1 tryptic peptide sequences were obtained by automated Edman degradation and used to design degenerate oligonucleotide primers for cDNA amplification by the reverse transcription–polymerase chain reaction

Figure 2. Mammalian Kip1 Sequences, and Comparison with Cip1/WAF1

(A) Amino acid sequences deduced from *Kip1* cDNAs from mink (mk), mouse (m), and human (h). Identical amino acids are indicated by elipses. The available mink sequence is incomplete at the C-terminus. Peptide sequences obtained from purified Kip1 are underlined. Thick underlining indicates the two sequences that served to design degenerate oligonucleotides for PCR.

(B) Sequence alignment between human Kip1 and Cip1/WAF1. The putative bipartite nuclear localization signal in both proteins is underlined. A Cdc2 kinase consensus site present in Kip1 is indicated by a thick bar.



Product	Relative Activity A:K2	E:K2	D2:K4
Kip1	100	100	100
Kip1(28-79)	25	5	1
Kip1(31-79)	0.1	0.03	0.1
Kip1(35-79)	<0.01	<0.01	<0.01
Kip1(28-64)	1.5	0.1	n.d.
Kip1(104-152)	<0.01	<0.01	<0.01

Figure 3. Cdk Inhibition by Kip1 In Vitro, and Identification of an Cdk Inhibitory Domain of Kip1

Cells lysates containing baculoviral cyclin E and Cdk2 (A and C) or the indicated cyclin/Cdk combinations were assayed for histone H1 kinase activity (A and B) and Rb kinase activity (C and D) in the presence of the indicated concentrations of Kip1. Representative gels containing the phosphorylated substrates are shown (A and C). Relative phosphorylation levels were quantitated, and are plotted as the percentage of phosphorylation observed in reactions without Kip1. (E) Schematic of the Kip1 protein, indicating the regions of highest homology to Cip1/WAF1 (closed boxes; see also Figure 2B). Bars and numbers indicate the size and location of the various fragments produced with a C-terminal hexahistidine tag and used in Cdk inhibition assays. The activity of these fragments is presented as a percentage relative to the activity of full-length Kip1.

(RT-PCR). A PCR product amplified out of reverse-transcribed Mv1Lu mRNA was used to screen a Mv1Lu cDNA library. This yielded one single positive clone that encoded the sequences obtained from the purified protein (Figure 2A). Screening of cDNA libraries from human kidney and mouse embryo with the *Kip1* cDNA yielded clones of highly related sequence. The human and mouse *Kip1* cDNAs had open reading frames of 594 bp and 591 bp, respectively, starting with an ATG codon in a favorable translation initiation context (Kato et al., 1994) and preceded by stop codons (data not shown). Compared with these open reading frames, the mink clone was incomplete and ended at nucleotide 534 (Figure 2A).

The *Kip1* cDNA encodes a predicted protein of 198 amino acids (22,257 daltons) in human and 197 amino acids (22,208 daltons) in mouse. These values are smaller than the 27 kDa value obtained with the purified mink protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To resolve this discrepancy, we constructed a cDNA encoding the mouse Kip1 sequence, tagged at the C-terminus with a hexahistidine sequence (~1 kDa mass). In vitro trancription and translation of this cDNA yielded a product that bound specifically to Ni^{2+}–NTA–agarose and migrated as a 28 kDa protein on SDS-polyacrylamide gels (see Figure 1C), confirming that the cloned cDNA encodes full-length Kip1 and that this protein migrates on SDS-polyacrylamide gels somewhat slower than its calculated molecular mass.

Kip1 Is Highly Conserved and Related to Cip1/WAF1

The predicted human, mouse, and mink Kip1 amino ac

sequences are highly related, showing ~90% identity (Figure 2A). A GenBank search revealed that, at the amino acid level, Kip1 shows significant homology only to Cip1/WAF1. The similarity was largely limited to a 60 amino acid segment in the N-terminal half of the protein. This region was 44% identical to the corresponding region in Cip1/WAF1 (Figure 2B). Like Cip1/WAF1, Kip1 has a putative bipartite nuclear localization signal (Dingwall and Laskey, 1991) near the C-terminus (Figure 2B). Yet unlike Cip1/WAF1, the Kip1 sequence does not have a putative zinc finger motif in the N-terminal region and has a C-terminal extension of 23 amino acids that contains a consensus Cdc2 phosphorylation site (Figure 2B).

Cdk Inhibitory Activity

Pure recombinant Kip1 tagged with hexahistidine at the C-terminus inhibited the histone H1 kinase activity of human cyclin A–Cdk2, cyclin E–Cdk2, and cyclin B1–Cdc2 complexes when assayed under linear reaction conditions (Figures 3A and 3B), whereas a mock sample from bacteria transformed with vector alone did not. Cyclin E–Cdk2 was inhibited half-maximally at 0.5 nM Kip1 (Figure 3B). Complete inhibition of cyclin A–Cdk2 required an 8-fold higher concentration, and this concentration was not sufficient to completely block cyclin B1–Cdc2 (Figure 3B). Addition of Kip1 to cyclin E–Cdk2, cyclin A–Cdk2, or cyclin D2–Cdk4 complexes inhibited their ability to phosphorylate a GST–Rb fusion product (Figures 3C and 3D). The relative sensitivity of cyclin E–Cdk2 and cyclin A–Cdk2 to inhibition by Kip1 in these assays paralleled their sensitivity in the histone H1 kinase assays (compare Figures 3B and 3D). Approximately 10 nM cyclin and 10 nM Cdk were used in these assays, but the actual concentration of cyclin:Cdk complexes is not known. Since Kip1 binds preferentially to cyclin–Cdk complexes (Polyak et al., 1994), the exact affinity and stoichiometry of their interaction with Kip1 remain to be determined.

Cdk Inhibitory Domain

We investigated whether the inhibitory activity of Kip1 resided in the region of similarity to Cip1/WAF1. A 52 amino acid peptide (Kip1[28–79]) corresponding to this region in Kip1 (Figure 3E) was produced recombinantly and purified with a C-terminal hexahistidine tag. This peptide inhibited Rb phosphorylation by cyclin A–Cdk2 with a potency that was close to that of full-length Kip1 (Figure 3E) and inhibited cyclin E–Cdk2 or cyclin D2–Cdk4 less effectively. Versions of this Kip1 region missing 3 amino acids at the N-terminus or 15 at the C-terminus were much weaker as Cdk inhibitors, and deletion of 7 N-terminal amino acids yielded a product with no inhibitory activity (Figure 3E). The peptide Kip1(104–152), which has little sequence similarity to Cip1/WAF1, was inactive as a Cdk inhibitor (Figure 3E).

Kip1 Prevents Cdk2 Activation

Kip1 was originally identified as a factor whose presence in extracts from quiescent cells rendered Cdk2 refractory to activation by phosphorylation at Thr-160. To determine whether Kip1 could block Cdk activation, we assayed its

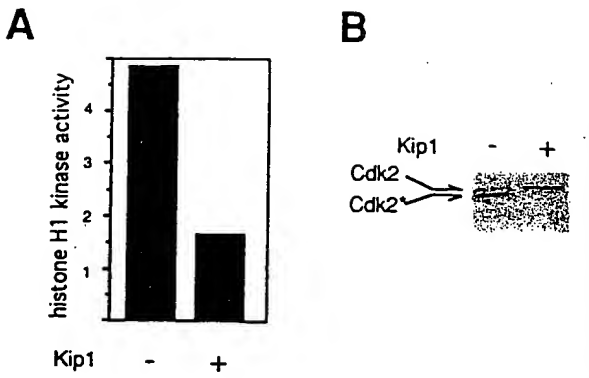


Figure 4. Kip1 Inhibits Activation of Cdk2 In Vitro

Extract from exponentially growing A549 cells were incubated with baculovirally expressed histidine-tagged cyclin E alone or together with Kip1. Cyclin E complexes was then retrieved with Ni^{2+}–NTA–agarose and assayed for histone H1 kinase activity (A), and by Western immunoblotting using anti-Cdk2 antibody (B). Kinase activity was quantitated by phosphorimager and expressed as arbitrary units. In (B), *Cdk2** indicates the faster migrating form of Cdk2 that corresponds to Cdk2 phosphorylated at Thr-160 (Gu et al., 1992).

effect on cyclin E–dependent Cdk2 activation in extracts from exponentially growing cells (Koff et al., 1993). A549 human lung carcinoma cell extracts were incubated with histidine-tagged cyclin E that was then retrieved and assayed for associated histone H1 kinase activity (Figure 4A). Addition of histidine-tagged Kip1 to the cell extracts markedly decreased the level of cyclin E–associated kinase activity (Figure 4A). In parallel assays, the retrieved cyclin E was subjected to SDS-PAGE and Western blotting with anti-Cdk2 antibodies. Cell extracts that did not receive Kip1 yielded cyclin E–associated Cdk2 in a form that corresponds to Cdk2 phosphorylated at Thr-160 (Gu et al., 1992) (Figure 4B). In contrast, cyclin E–associated Cdk2 from extracts that received Kip1 was exclusively in the inactive form (Figure 4B). Collectively, these results suggested that Kip1 binding to preactive cyclin E–Cdk2 complexes in vitro prevented Thr-160 phosphorylation and activation of Cdk2.

Kip1 Overexpression Inhibits Cell Entry into S Phase

Mouse *Kip1* subcloned into a mammalian expression vector was transfected into Mv1Lu cells under conditions in which up to 65% of the cell population takes up and transiently expresses transfected plasmids (Attisano et al., 1993). The rate of [^{125}I]deoxyuridine incorporation into DNA was reduced 70% in cells transfected with *Kip1* compared with cells transfected with vector alone (Table 1). To determine the effect on cell cycle distribution, *Kip1* was cotransfected with a *CD16* expression vector (Kurosaki and Ravetch, 1989) that allowed flow cytometric separation of the transfected cells based on CD16 immunofluorescence. The $CD16^+$ population cotransfected with *Kip1* showed a larger proportion of cells in G1 phase and a smaller proportion in S phase than the $CD16^+$ population cotransfected with vector alone (Table 1), suggesting that

Table 1. Kip1 Blocks Entry into S Phase

Time after Transfection	Vector	[^{125}I]Deoxyuridine Incorporation[a]	Percentage of CD16$^+$ Cells in S Phase[b]
24 hr	pCMV5	27,581 ± 5,126	26 ± 5
	pCMV5-Kip1	8,386 ± 1,250	9 ± 2
48 hr	pCMV5	5,126 ± 47	35 ± 2
	pCMV5-Kip1	1,510 ± 140	7 ± 1

The TGFβ receptor–defective R-1B cell line was cotransfected with a human *CD16* expression vector and pCMV5 or pCMV5 containing the mouse *Kip1* cDNA. Assays were conducted at the indicated times after transfection.

[a] [^{125}I]deoxyuridine incorporated over a 3 hr period by the entire cell population. Data are the average ± SD of triplicate determinations.

[b] Transfected cells were immunostained with anti-CD16 and analyzed for DNA content. Data are the average of two separate experiments and show the range of values.

Kip1 overexpression obstructed cell entry into S phase. Cell numbers after transfection indicated that *Kip1* did not cause cell death (data not shown).

Kip1 mRNA Distribution and Levels in Quiescent and Proliferating Cells

The level of endogenous *Kip1* mRNA expression in various human tissues was determined by Northern blot analysis. The only mRNA detected was a species of 2.5 kb present at similar levels in all tissues tested, although it was somewhat higher in skeletal muscle and lower in liver and kidney (figure 5A). *Kip1* mRNA levels were similar in exponentially proliferating and contact-inhibited Mv1Lu cells and did not change when cells were released from contact inhibition by being plated at low density in the presence of serum (Figure 5B). Addition of TGFβ to cells released from contact inhibition also did not affect *Kip1* mRNA levels (Figure 5B). These results indicated that the regulation of Kip1 by extracellular antiproliferative signals occurs at a posttranscriptional level.

Discussion

A Family of Cdk Inhibitors

Human *Kip1* encodes a protein of 198 amino acids that is highly conserved (~90% identity) in mouse and mink. Its most distinctive feature is a 60 amino acid region in the N-terminal half that has amino acid sequence similarity to Cip1/WAF1 (El-Deiry et al., 1993; Harper et al., 1993; Noda et al., 1994; Xiong et al., 1993). Like Cip1/WAF1, Kip1 contains a potential nuclear localization signal in the C-terminal region. In Kip1, this region also contains a consensus Cdc2 kinase site that might play a role in feed-back regulation by their target kinases.

The structural similarity between Kip1 and Cip1/WAF1 defines a family of mammalian Cdk inhibitors with different

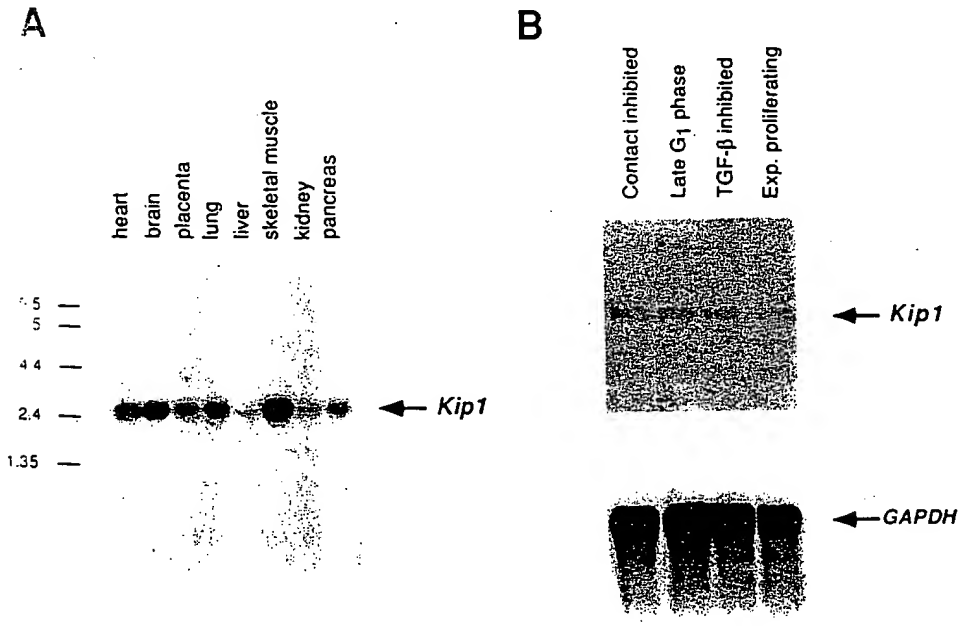


Figure 5. Expression Pattern of Kip1 in Various Tissues and Cell Proliferation States

Kip1 Northern blots using equal amounts of poly(A)$^+$ RNA from the indicated human tissues (A) or from Mv1Lu cells in different proliferation states (B). The latter blot was rehybridized with a glyceraldehyde-phosphate dehydrogenase probe.

regulatory properties. Kip1 is involved posttranscriptionally in the action of extracellular signals (this work), and its silencing in exponentially growing cells correlates with binding to a heat-labile component (Polyak et al., 1994). In contrast, Cip1/WAF1 is regulated transcriptionally by p53, senescence, and cell quiescence (Dulić et al., 1994; El-Deiry et al., 1994; Noda et al., 1994). Kip1 and Cip1/WAF1 are more effective against G1 Cdks than against mitotic Cdks. However, Kip1 was more effective against cyclin E–Cdk2 than against cyclin A–Cdk2 (or cyclin D2–Cdk4), whereas in similar assays, Cip1/WAF1 was more effective against cyclin A–Cdk2 (Harper et al., 1993). Kip1 effectiveness is likely defined by its binding affinity for a given cyclin–Cdk complex (Polyak et al., 1994). These binding constants, and the differences in Cdk specificity with Cip1/WAF1, remain to be assessed with purified cyclin–Cdk complexes.

The Kip1 region that is similar to Cip1/WAF1 is sufficient to inhibit Cdk activity when tested as a 52 amino acid peptide in vitro. This 52 amino acid segment contains the sequence LFGPVN, which corresponds to the longest uninterrupted stretch of identity to Cip1/WAF1 and, interestingly, is similar to the FAR1 sequence LSQPVN located in a region required for interaction with CLN2–CDC28 (Peter et al., 1993).

Cdk Inhibition at Two Levels

Kip1 can inhibit both the process of Cdk activation and the kinase activitiy of cyclin–Cdk complexes assembled and activated in intact cells. Kip1 was originally identified as a factor whose presence in extracts of quiescent cells rendered them unable to activate Cdk2 by phosphorylation at Thr-160 (Polyak et al., 1994). Indeed, recombinant Kip1 inhibits Cdk2 Thr-160 phosphorylation and activation in vitro. Although Kip1 could act as an inhibitor of the Cdk-activating kinase, previous results tend to argue against this possibility (Polyak et al., 1994). The dual effects of Kip1, on Cdk2 activation and Cdk2 activity, might relate to the fact that Thr-160 is located in a loop that closes the substrate-binding cleft in the Cdk2 structure (DeBondt et al., 1993). It is conceivable that binding of Kip1 to this region might interfere with Thr-160 phosphoryiation as well as with the catalytic function of activated Cdk2.

Function in the Cell Cycle

Cyclin E–Cdk2 and cyclin D–Cdk4 are rate limiting for G1 progression (Jiang et al., 1993; Ohtsubo and Roberts, 1993; Quelle et al., 1993). Inhibition of these kinases by Kip1 in vivo would render cells unable to reach that transition. The strong reductions in the rate of DNA synthesis and the proportion of cells in S phase caused by *Kip1* transfection are consistent with this possibility and with a role of Kip1 as mediator of extracellular growth-inhibitory signals.

As cells released from contact inhibition move closer to S phase, their extracts contain progressively lower levels of Kip1 activity, and this decline can be prevented by TGFβ addition early in G1 phase (Polyak et al., 1994). However, the present results show that contact-inhibited cells and TGFβ-treated cells have *Kip1* mRNA levels equal to those of proliferating cells. Furthermore, extracts from proliferating cells yield active Kip1 when they are heated transiently at 100°C (Polyak et al., 1994). One interpretation of these observations is that Kip1 is progressively sequestered by binding to a heat-labile component as cells progress through G1, and this process can be prevented by TGFβ. Mitogens and antimitogens might regulate Kip1 activity or availability by controlling its binding to a silencing protein. Alternatively, Kip1 might be a passive regulator whose uniform levels could ensure that active Cdks become available only when their levels reach the threshold imposed by binding to Kip1. In the latter situation, even small effects of mitogens and antimitogens on cyclin or Cdk protein levels could become amplified by the existence of that threshold.

The present isolation of *Kip1* cDNAs will facilitate the study of its function and regulation in the cell cycle, its possible role as a determinant of cell susceptibility to growth inhibition by TGFβ, and its possible implication in the loss of inhibition by TGFβ and cell contact often observed in oncogenically transformed cells.

Experimental Procedures

Metabolic Labeling, Immunoprecipitations, and Peptide Mapping

Mv1Lu cells were synchronized by contact inhibition, treated with TGFβ, metabolically labeled, lysed, and immunoprecipitated with anti-Cdk2 antibody or chromatographed on cyclin E–Cdk2 affinity columns, according to Polyak et al. (1994). For peptide mapping, the 27 kDa band present in Cdk2 immunoprecipitates and in the cyclin E–Cdk2 affinity column eluates was cut out from the gels, digested with 0.1 μg of V8 protease, resolved on 15%–22.5% gradient gels.

Baculoviral Proteins

The human cyclin E cDNA (Koff et al., 1991) was tagged at the N-terminus with a hexahistidine sequence. This cDNA was cloned into baculovirus transfer vector pVL1392 and expressed in Sf9 cells as described in the BaculoGold Transfection Kit (Pharmingen). Baculoviruses encoding other cyclins and Cdks were provided by C. Sherr and H. Piwnica-Worms. Baculoviral proteins were prepared by the method of Desai et al. (1992).

Kip1 Purification

Two hundred 150 mm dishes of contact-inhibited Mv1Lu cells (~2 × 10^{10} cells) were collected by trypsinization and lysed in hypotonic buffer by sonication. The extracts were clarified by centrifugation, heated to 100°C for 5 min, and clarified by centrifugation. Agarose-precleared extracts were allowed to bind to histidine–cyclin E–Cdk2 complexes immobilized on Ni^{2+}–NTA–agarose (Polyak et al., 1994). Specifically bound proteins were eluted with 6 M guanidium hydrochloride solution, dialyzed overnight against 1 × HBB buffer (25 mM HEPES–KOH [pH 7.7], 150 mM NaCl, 5 mM $MgCl_2$, 0.05% NP-40, and 1 mM DTT) (Kaelin et al., 1992), and acetone precipitated.

Protein Sequence Analysis

Protein was fractionated by SDS-PAGE and electroblotted onto nitrocellulose, and the Ponceau S–stained 27 kDa band was excised and processed for internal amino acid sequence analysis (Tempst et al., 1990). HPLC peak fractions (over trypsin background) were analyzed by a combination of automated Edman degradation and matrix-assisted laser desorption (MALDI-TOF) mass spectrometry (Elicone et al., 1994). Mass analysis (on 2% aliquots) was carried out using a model LaserTec Research MALDI-TOF instrument (Vestec) and α-cyano-4-hydroxy cinnamic acid as the matrix. Chemical sequencing (on 95% of the sample) was done using an Applied Biosystems 477A sequenator optimized for femtomole level analysis.

Kip1 cDNA Cloning and Northern Blot Analysis

RT–PCR reactions were performed using degenerate oligonucleotides as primers and total RNA from contact-inhibited Mv1Lu cells as template. The combination of one pair of primers (see Figure 2A) yielded a 135 bp fragment that was used to screen a λZAPII cDNA library prepared from Mv1Lu cells (Pines, 1994). The mouse *Kip1* cDNA was obtained from a λEXlox mouse embryo cDNA library (Novagen), and human *Kip1* cDNA was obtained from a λgt11 kidney cDNA library (Clontech). Poly(A)$^+$ RNA blots were hybridized with a PCR-derived fragment of the mouse *Kip1* cDNA labeled by random priming.

In Vitro Translation

A NdeI–XhoI fragment containing the coding region of the mouse *Kip1* cDNA (nucleotides 1–591) was subcloned to pCITE2a (Novagen). This construct encodes a fusion protein containing a C-terminal hexahistidine sequence and 6 amino acids from the vector at the N-terminus of Kip1. In vitro transcription and translation were performed using Red Nova lysate (Novagen).

Recombinant Kip1

591 bp PCR generated NheI–XhoI fragment of the mouse *Kip1* cDNA containing the full-length coding region was subcloned into pET21a (Novagen), yielding a construct that encodes Kip1 with a C-terminal hexahistidine sequence. The protein was expressed in BL21(DE3) bacteria and purified by sonicating cells in a solution containing 8 M urea, 50 mM Tris–HCl (pH 7.4), 20 mM imidazole, clarified by centrifugation and bound to Ni^{2+}–NTA agarose for 1 hr at 4°C. The column was washed with a 6–0.75 M urea reverse gradient in 0.5 M sodium chloride, 50 mM Tris (pH 7.4), and 20% glycerol and eluted with 200 mM imidazole, 20 mM HEPES (pH 7.4), 1 M KCl, 100 mM EDTA. The eluate was dialyzed overnight against 1 × HBB buffer and stored at −70°C until use.

In Vitro Kinase and Cdk2 Activation Assays

H5 insect cell extracts containing baculovirally expressed cyclins and Cdks were incubated with recombinant Kip1 for 30 min at 37°C, precipitated with anti-HA antibody, and the histone H1 kinase activity of these complexes was assayed (Koff et al., 1993). Rb kinase reactions were done according to Matsushime et al. (1992). The phosphorylation of the histone H1 band and Rb band were quantitated with a phosphorimager (Molecular Dynamics).

Hypotonic cell extracts from exponentially growing A549 cells were incubated with baculoviral histidine–cyclin E protein, with or without Kip1, at 37°C for 30 min. Mixtures were then diluted 10-fold in 1 × NP-40 RIPA buffer containing 20 mM imidazole and incubated with Ni^{2+}–NTA–agarose at 4°C for 1 hr. One portion of the samples was run using 12% SDS-PAGE and immunoblotted with anti-Cdk2 antibody (Koff et al., 1993).

Kip1 Transfections and Flow Cytometry Analysis

The mouse *Kip1* cDNA (nucleotides −82 to +591) was subcloned into pCMV5 (Attisano et al., 1993). R-1B cells were cotransfected with 0.5 μg/ml of pCEXV-3 containing murine *CD16* cDNA (Kurosaki and Ravetch, 1989) and 3 μg/ml of pCMV5 alone, or with 3 μg/ml of pCMV5-Kip1 (Attisano et al., 1993). CD16 immunostained cells (Wirthmueller et al., 1992) were analyzed by flow cytometry using FACScan (Becton Dickinson) and Multicycle software (PHOENIX Flow Systems).

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GenBank Accession Numbers

The numbers for the sequences reported in this paper are U09966 (mink), U09968 (mouse), and U10906 (human).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: H. Lowenheim — Attorney Docket No.: SOPH116953

Application No.: 09/622,719 — Group Art Unit: 1635

Filed: October 18, 2000 — Examiner: K.A. Lacourciere

Title: METHOD FOR THE TREATMENT OF DISEASES OR DISORDERS OF THE INNER EAR

THIRD DECLARATION OF JONATHAN KIL

Seattle, Washington 98101

January 4, 1904

TO THE COMMISSIONER FOR PATENTS:

I, Jonathan Kil, declare as follows:

1. I am the Chief Executive Officer of Sound Pharmaceuticals, Inc., Seattle, Washington, and I am familiar with the subject matter disclosed and claimed in the above-identified application.

2. My colleagues and I conducted the following experiments to assess the effect of a variety of antisense oligonucleotides on the level of expression of $p27^{Kip1}$ mRNA in mouse NIH 3T3 cells cultured *in vitro*.

3. NIH 3T3 cells were transfected with the 14 antisense oligonucleotides shown in Table 1. The antisense oligonucleotides corresponded to portions of the target $p27^{Kip1}$ mRNA. The location of each oligonucleotide is given with reference to the sequence of the mouse $p27^{Kip1}$ cDNA (GenBank accession number U09968; reported in Polyak,K., et al, *Cell* **78:** 56-66 (1994)). Antisense oligonucleotides SPI5114 and SPI5116 have identical nucleic acid sequences, but SPI5116 includes both phosphothioate and 2" MOE backbone linkages.

Table 1

Oligonucleotide Name	Oligonucleotide Sequence	Location of Oligonucleotide
SPI5101	TGGCTCTCCTGCGCC	306-320

Attachment A

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Oligonucleotide Name	Oligonucleotide Sequence	Location of Oligonucleotide
SPI5108	CATCCTGGCTCTCCTGCGCCAGCAC	301-325
SPI5114	CCGCTGACATCCTGGCTCTCCTGCG	308-332
SPI5116	CCGCTGACATCCTGGCTCTCCTGCG	308-332
SPI5906	TCTCACGTTTGACAT	1-15
SPI5907	ATTCCACTTGCGCTG	169-183
SPI5908	TCTCCACCTCCTGCC	227-241
SPI5501	TGCTCCGCTAACCC	247-440
SPI5505	GACACTGCTCCGCTAACCCAGCCTG	421-445
SPI5517	GACACTGCTCCGCTAACCCAG	425-445
SPI5518	CTGCTCCGCTAACCCAGCCTG	421-441
SPI5519	CACTGCTCCGCTAACCCAGCC	423-443
SPI5801	CATCCGCTCCAGGCT	34-48
SPI5806	CGTCCATCCGCTCCAGGCTCG	32-52

The cells were incubated in the presence of the oligonucleotide for 26 hours. Real time RT-PCR was used to measure the amount of $p27^{Kip1}$ mRNA present in total RNA extracted from the treated cells.

4. Enclosed herewith as Attachment B is a graph showing the level of $p27^{Kip1}$ mRNA in the cells treated with the different oligonucleotides, compared to the control level of $p27^{Kip1}$ mRNA in cells treated with the Lipofectamine lipid delivery vehicle without oligonucleotides. The results shown in the graph demonstrate that all of the tested oligonucleotides caused a significant reduction in the level of $p27^{Kip1}$ mRNA in the treated cells.

5. All statements made herein and of my own knowledge are true, and all statements made on information and belief are believed to be true; and further, these statements were made

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with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,

Jonathan Kil, M.D.

Date: 12/27/04

BFM:tmm

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Education:

University of Virginia	M.D.	1992-1996
Georgetown University	MD/PhD (candidate)	1990-1992
University of California, Irvine	B.S. in Biological Sciences	1985-1989

Experience:

2001-	Founder, President, CEO Sound Pharmaceuticals Inc., Seattle, WA
1998-2001	Founder, President, CEO, CSO Otogene USA, Inc., Seattle, WA Member of the Vorstand, Otogene AG, Tuebingen, Germany
1996-1998	Senior Fellow Department of Otolaryngology-HNS and VM Bloedel HRC University of Washington
1992-1996	M.D./Ph.D. (candidate) Departments of Neurosciences and Otolaryngology-HNS University of Virginia (UVA)
1990-1992	M.D./Ph.D. candidate (transferred to UVA to conduct inner ear research) Departments of Cell Biology and Otolaryngology-HNS Georgetown University
1989-1990	Research Assistant Department of Anatomy and Neurobiology University of California, Irvine

Grants:

2000-2002	NIH SBIR Phase II #DC04258-02, P.I.
1999-2000	NIH STTR Phase I #DC04258-01, P.I.
1996-1998	Individual NRSA Postdoctoral Research Fellowship #DC00247, P.I.
1991-1992	American Hearing Research Foundation Research Grant, Co-investigator

Awards/Honors:

1995	Association for Research in Otolaryngology Medical Student Travel Award
1995	Winn Medical Student Scholarship for Otolaryngology-HNS
1991	Achievement Reward for College Scientists (ARCS) Foundation Scholarship
1989	Ralph W. Gerard Award for Outstanding Research
1989	Excellence in Research Honors

Publications:

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Kil, J., Hanigan, M. H., Taylor, Jr., P.T. and Hashisaki, G.T. (1996) Localization of gamma-glutamyl transpeptidase in the chick inner ear sensory epithelia. Soc. Neuro. Abs. 22, 1622.

Memberships:
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Attachment B